PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification [4] :		(11) International Publication Number:
C12N 5/00	A1	WO 89/ 07135
		(43) International Publication Date: 10 August 1989 (10.08.89)

(21) International Application Number: PCT/US88/04700

(22) International Filing Date: 29 December 1988 (29.12.88)

(31) Priority Application Number: 149,427

(32) Priority Date: 29 January 1988 (29.01.88)

(33) Priority Country: US

(71) Applicant: GRANADA GENETICS, INC. [US/US]; 100 Research Parkway, Texas A&M University Research Park, College Station, TX 77840 (US).

(72) Inventor: BONDIOLI, Kenneth, R. ; 2332 Westwood Main, Bryan, TX 77801 (US).

(74) Agent: BOULWARE, Margaret, A.; One Riverway, Suite 1100, Houston, TX 77056 (US).

(81) Designated States: AT (European patent), AU, BE (European patent), CH (European patent), DE (European patent), FR (European patent), GB (European patent), IT (European patent), JP, LU (European patent), NL (European patent), SE (European patent).

Published
With international search report.
Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(54) Title: BOVINE EMBRYO IN VITRO CULTURE

(57) Abstract

A culture method and system has been realized for *in vitro* development of bovine embryos from early stage 1- to 2-cell embryos to morula or blastocyst stage. The embryos are placed in a culture system of a buffered balanced salt solution with a carbohydrate energy source medium and bovine oviductal epithelial cells in suspension. Embryo development is enhanced by the addition of bovine or ovine oviductal fluid as well as non-essential amino acids to the system. The method and system can be used to culture genetically manipulated embryos used in transgenic procedures and nuclear transfer.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria
AU	Australia
BB	Barbados
BE	Belgium
BG	Bulgaria
BJ	Benin
BR	Brazil
CF	Central African Republic
CG	Congo
CH	Switzerland
CM	Cameroon
DE	Germany, Federal Republic of
DK	Denmark
FI	Finland
FR	France
GA	Gabon
GB	United Kingdom
HU	Hungary
IT	Italy
JP	Japan
KP	Democratic People's Republic of Korea
KR	Republic of Korea
LI	Liechtenstein
LK	Sri Lanka
LU	Luxembourg
MC	Monaco
MG	Madagascar
ML	Mali
MR	Mauritania
MW	Malawi
NL	Netherlands
NO	Norway
RO	Romania
SD	Sudan
SE	Sweden
SN	Senegal
SU	Soviet Union
TD	Chad
TG	Togo
US	United States of America

BOVINE EMBRYO IN VITRO CULTURE

Background of the Invention

The in vitro culture of embryos of domestic animals has been studied and is of interest particularly with new techniques of genetic manipulation of early stage embryos. In vitro culture involves removing an egg from the animal's reproductive tract. Fertilization can occur before or after removal. Later stage embryos are also cultured in vitro which have been removed from the animal at a time after fertilization. The removed embryos are then placed in a culture medium for further development.

A number of domestic and laboratory animal embryos have been used for in vitro culture experiments. Wright and Bondioli, "Aspects of In Vitro Fertilization and Embryo Culture in Domestic Animals," J. Anim. Sci., vol. 53, No. 3, pp.702-729 (1981). The embryos have been removed at very early stages, 1- to 2-cell, and later uterine stages, around 32-cell, and cultured in vitro. Generally a high proportion of the later stage embryos from domestic and laboratory animals will develop in vitro to a blastocyst stage and many will hatch from the zona pellucida.

In bovine in vitro culture a "blocking" phenomenon has been observed at the 8- to 16-cell stage when culturing from 1-cell embryos. Kane, "Culture Media and Culture of Early Embryos," Theriogenology, vol. 27, No. 1, pp. 49-57 (1987); Eyestone and First, "A Study of the 8- to 16-Cell Developmental Block in Bovine Embryos Cultured In Vitro," Theriogenology, vol. 25, No. 1, p. 152 (1986). The bovine embryos generally fail to develop past the 8- to 16-cell stage to the blastocyst stage when cultured in tissue culture media with various supplements. In one study 1- and 8-cell stage embryos were cultured in various media. The

8-cell embryos were successfully cultured to blastocyst stage *in vitro* in selected medium, however none of the 2-cell embryos underwent more than one complete cell division *in vitro*. Wright et al., "Successful Culture *In Vitro* of Bovine Embryos to the Blastocyst Stage, *Biol. Reprod.*, vol. 14, pp.157-162 (1976). In another study, three of twenty-two 1- to 2-cell stage bovine embryos cultured in medium developed into blastocysts, but this work has not been reportedly reproduced. Wright et al., "Blastocyst Expansion and Hatching of Bovine Ova Culture *In vitro*," *J. Anim. Sci.*, vol. 43, p.170 (1976).

The conventional method of culturing bovine embryos which have been removed at the 1- to 2-cell stage is to transfer the embryo to a sheep oviduct for *in vivo* culture which overcomes the blocking observed *in vitro*. The embryos are later flushed from the sheep oviduct and transferred to a recipient cow. The conventional method of bovine embryo culture involves the additional steps of culturing for a period of time in sheep, removal from sheep and re-introduction into the recipient cow. During the course of the transfers, there can be damage to or loss of the embryos.

Another alternative for culturing bovine embryos is a co-culture system which includes the use of a layer of cells cultured from embryonic or adult tissue with a culture medium. The co-culture technique has promoted successful development for early stage embryos. However, the success rate for development to blastocyst or morula is higher when culturing embryos older than 1- to 2-cell. Camous et al, "Cleavage Beyond the Block Stage and Survival After Transfer of Early Bovine Embryos Cultured With Trophoblastic Vesicles", *J. Reprod. Fert.*, vol. 72, pp. 479-485 (1984). In some cases, co-culture systems have been used only with bovine embryos at the 4-cell and older stages. Eyestone et al, "Co-Culture of Early Bovine Embryos With Oviductal Epithelium", *Theriogenology*, vol. 27, No. 1, p. 228 (1987).

Summary of the Invention

The present technique and components realize a system which cultures in vitro a substantial percentage of early stage bovine embryos to morula and blastocyst stage. The system uses a culture medium of buffered balanced salt solution with a carbohydrate energy source and bovine oviductal epithelial cells in a suspension. The early stage, 1- to 2-cell embryos are collected and placed in a drop of the medium with about 5% to 15% volume of cells in the fluid medium.

The culture system drop is covered under a film such as silicone or paraffin oil. The suspension system is incubated at about 38°C. in a 5% CO_2 and 95% air humidified atmosphere. The cells develop to blastocyst stage in about seven days. At this point the embryos can be transferred to a recipient cow by non-surgical means.

The culture medium can be supplemented with amino acids, growth factors and vitamins. The addition of non-essential amino acids increases the development rate of the embryos.

The culture system may be supplemented with oviductal fluid from bovine or ovine. The oviductal fluid is mixed with the culture medium. A 1:1 ratio of culture medium and oviductal fluid with the bovine oviductal epithelial cells system produces a substantial percentage of early stage embryos developing into blastocysts.

The system of this invention can be used for bovine embryos and eggs that are subject to genetic research. In the nuclear transplantation studies, the genetic material from a selected cell is transferred with an egg that has been subjected to an enucleation procedure. The egg with the new genetic material is fused and cultured. Using the system of this invention, the culture process can be in vitro and the embryos can be observed during the critical early stages. For the nuclear transfer procedures, a system with demonstrated success for culturing 1-cell through blastocyst is a necessity. After the embryos are cultured, they may be transferred to recipient cows as desired.

Similarly for research involving gene transfer, an in vitro system which is successful with early stage embryos is needed. In gene transfer work, foreign DNA is inserted into the nuclear material of preferably 1-cell embryos. A culturing method is then used to grow the embryo to determine whether a transgenic embryo develops with the foreign gene incorporated and expressed. An in vitro technique would allow the investigator to observe which embryos were developing and make further determination of the progress of culture.

Description of the Preferred Methods and Embodiment

The following is a description of the preferred method and alternatives for culturing in vitro bovine embryos. The procedures have been used to culture early stage embryos from 1- and 2-cell until development to the morula or blastocyst stage. The techniques were utilized with early stage embryos used for transgenic research, the introduction of foreign DNA with the bovine embryo, and nuclear transfer research wherein nuclear donor material was transferred to an enucleated egg. The method was also used to develop bovine embryos which were not subject to any genetic manipulation.

The method is successful for in vitro culture of manipulated and non-manipulated bovine embryos from the 1-cell stage to past the 8- to 16-cell stage.

The preferred method and culture system utilizes a buffered balanced salt solution with a carbohydrate energy source medium. The balanced salt solution includes inorganic salts maintained at a physiological pH by a bicarbonate -CO_2 buffer system. The carbohydrate energy source can include glucose, lacate, pyruvate or acetate. A medium of choice was Brinsters Mouse ova culture medium modified by the addition of sodium acetate and supplemented with bovine serum albumin. The medium was also supplemented with essential amino acids, non-essential amino acids and a combination of essential and non-essential amino acids. The

components of the medium with non-essential amino acids included are listed in Table 1.

Table 1

PREFERRED CULTURE MEDIUM
FOR IN VITRO CULTURE OF BOVINE EMBRYOS

Component	Grams/Liter Unless Otherwise Noted
NaCl	5.546
KCl	.356
$CaCl \cdot 2H_2O$	.250
KH_2PO_4	.162
$MgSO_4 \cdot 7H_2O$	.294
$NaHCO_3$	2.106
Glucose	1.000
Na Lactate	3.6 ml of 60% syrup
Na Pyruvate	.056
EDTA	.034
Na Acetate	.050
Phenol Red	1 ml of .05% solution
L-Alanine*	8.9 mg/l
L-Asparagine H_2O*	15.0 mg/l
L-Aspartic Acid*	13.30 mg/l
L-Glutamic Acid*	14.70 mg/l
Glycine*	7.5 mg/l
L-Proline*	11.50 mg/l
L-Serine*	10.50 mg/l
bovine serum albumin	6.0 mg/l
penicillin	10,000 units/l
streptomycin	10,000 mcg/l

*Non-essential amino acids.

The basic culture medium was the components listed in Table 1 less the non-essential amino acids.

The preferred method utilizes bovine oviductal epithelial cells in the culture system which are recovered

from oviductal flushings when early stage embryos are recovered. The cells chosen are predominantly ciliated epithelial cells. The oviductal ciliated epithelial cells are washed once in phosphate buffered saline by allowing the clumps of cells to settle in a tube. The cells were then washed twice in culture media by pipetting. The cells were generally used the same day of collection. The cells have been frozen and stored in liquid nitrogen and used successfully after storage.

The culture system can be comprised of a mixture of the buffered balanced salt with a carbohydrate energy source medium and the cells. A 25 µl drop of medium will have about 5% to 15% by volume of cells or about 2 to 4 µls of cells added to the drops in a suspension-type system.

In the preferred method oviductal fluid collected from a cow or sheep is part of the culture system along with the medium and cells. In some cases, a superovulated cow which had occulted oviducts produced a large amount fluid which had accumulated. A superovulated animal was ligated surgically on one or both ends of the oviduct and fluid was recovered. The bovine oviductal fluid (BOF) was recovered in a sterile manner. Any cellular debris was removed by centrifugation. The BOF can be frozen until utilized. Sheep oviductal fluid has been used with some success.

In the preferred method and preferred culture system a 1:1 mixture of medium described in Table 1 and BOF is used. The amount of BOF can be decreased since medium alone can be used. A culture drop of 25 µl of the mixture with 2 µl of loosely packed cells added make up a drop of the culture system.

The embryos were placed in a drop of the culture system. Preferably 1 to 10 embryos are contained in one drop. The embryos were washed once in a drop of the culture mixture before being placed in the culture drop. The culture system drop is covered with a film of paraffin, silicone oil or other inert covering. The culture system with embryos is kept at about 38°C under a 5% CO_2 and 95% air humidified system.

Larger volume culture drops of 1 ml or higher can be used. With a larger culture drop the oil covering can be omitted if desired. The oil covering is used to prevent evaporation of medium from a small volume.

The culture is maintained for seven days during which time the normally developing embryos will be at the morula or blastocyst stage. The embryos can be transferred non-surgically to recipient cows for full development. The development to blastocysts, approximately 60 to 100 cells, exceeds the previously reported "blocking" in bovines at the 8- to 16-cell stage.

This procedure has been used to culture embryos that have not been the subject of any manipulation to determine the successful development from 1-cell to blastocyst stage. Separate experiments were run with combinations of medium (as shown in Table 1 with and without the non-essential amino acids listed therein), BOF and cells in the culture system. In these experiments, it is not possible to determine at a 1-cell stage whether fertilization has been successful so that failure of development due to lack of fertilization can occur. The following Table 2 shows the results of the *in vitro* method and various culture systems. The development was terminated after seven days of culture to record the cellular count.

Table 2

CULTURE SYSTEM RESULTS

	Medium Only	Medium, Cells	Medium, BOF	Medium, BOF, Cells
Number of Embryos	35	35	35	35
Percentage Developing After 7 Days	3%	43%	17%	60%

The results show the development to the morula or blastocyst stage was greatest in the mixture of medium, BOF, and cells with 60% of the embryos developing. A system of medium and the cells showed a 43% development to morula or blastocyst stage.

The culture medium was varied to include combinations of essential and non-essential amino acids, vitamins and other additives as described below.

A culture medium with the basic components of Table 1 (components of Table 1 less the non-essential amino acids) plus all of the essential and non-essential amino acids listed in Table 3 below was prepared.

Table 3

COMBINED ESSENTIAL AND NON-ESSENTIAL AMINO ACIDS

Amino Acid	mg/l of medium
L-alanine	9.0
L-arginine HCl	211.0
L-asparagine-H_2O	15.01
L-aspartic acid	13.0
L-cysteine	25.0
L-glutamic acid	14.7
L-glutamine	146.0
Glycine	7.51
L-histidine HCl-H_2O	23.0
L-isoleucine	2.6
L-leucine	13.0
L-lysine HCl	29.0
L-methionine	4.48
L-phenylalanine	5.0
L-proline	11.5
L-serine	10.5
L-threonine	3.57
L-tryptophan	.6
L-tyrosine	1.81
L-valine	3.5

The basic culture medium was also supplemented with vitamins and other additives as shown in Table 4.

Table 4

MEDIUM SUPPLEMENTS VITAMINS + OTHER ADDITIVES

	mg/l
Hypoxanthine	4.0
Lipoic acid	.2
Thymidine	.7
Biotin	.024
D-Ca:Pantothenate	.715
Choline chloride	.698
Folic acid	1.320
Inositol	.541
Niacinamide	.615
Pyridoxine-HCl	.206
Riboflavin	.376
Thiamine	1.0
Vitamin B_{12}	1.36

Hypoxanthine, lipoic acid and thymidine are not generally considered vitamins but were included in this mixture referred to as a vitamin mixture. Hypoxanthine, a nucleotide precursor; thymidine, a nucleotide and lipoic acid, a coenzyme were also added.

Systems using various culture media were tested with oviductal cells in suspension but without BOF. The media used were: (1) basic medium without any amino acids, (2) basic medium with vitamins (Table 4), (3) basic medium with all amino acids in Table 3, and (4) basic medium with vitamins (Table 4) and all amino acids in Table 3. The results are reported in Table 5 showing the number of embryos developing to the morula or blastocyst stage.

Table 5

	(1) No Amino Acids Medium	(2) Medium + Vitamins	(3) Medium + Amino Acids	(4) Medium + Vitamins + Amino Acids
Number of Embryos	31	35	33	32
Number Developing	12	13	24	20
Percentage Developing	39%	37%	73%	63%

The results indicated that the development was improved when the medium is supplemented with amino acids.

Additional culture systems were prepared using media supplemented with various amino acid mixtures and cells. The basic medium has the components listed in Table 1 less the non-essential amino acids. The various media tested were the basic medium of Table 1 plus essential and non-essential amino acids of Table 3. A medium was prepared with the basic medium of Table 1 and the essential amino acids used in MEM medium as shown in Table 6 below.

Table 6

MEM ESSENTIAL AMINO ACIDS

Amino Acids	mg/l
L-arginine HCl	126.40
L-cystine	24.0
L-histidine HCl-H_2O	42.00
L-isoleucine	52.50
L-leucine	52.40
L-lysine HCl	72.50
L-methionine	15.10
L-phenylalanine	33.00
L-threonine	47.60
L-tryptophan	10.20
L-tyrosine	36.00
L-valine	46.80

The culture medium of Table 1 includes the non-essential amino acids which are used in MEM tissue culture medium. Another medium was prepared combining the MEM essential and

non-essential amino acids of Tables 1 and 6 with the other basic medium components of Table 1.

The culturing method as described herein was followed using the various media and oviductal cells in the suspension system. Table 7 records the results of culture in vitro to morula or blastocyst stage for bovine embryos.

Table 7

CULTURE RESULTS AMINO ACID SUPPLEMENTS

	Basic Medium + Amino Acids Table 3	Medium Table 1	Basic Medium + MEM Essential Amino Acids Table 6	Basic Medium + All MEM Amino Acids Tables 1-6
Number of Embryo	26	27	27	25
Number Developing	10	19	9	11
Percentage Developing	39%	70%	33%	44%

The medium of Table 1 with the non-essential amino acids of MEM medium exhibited the best results.

The in vitro development technique has been used for bovine embryos that have been subject to transgenic procedures. In these cases the pronucleus of a presumably fertilized embryo was injected with various foreign DNAs to determine if the foreign gene could be incorporated and expressed. The injection of foreign DNA can be lethal to the embryo. Also, not all embryos are fertilized. The injected embryos were cultured according to the preferred method in a system including medium of Table 1 in a 1:1 ratio with BOF and about 2 µl of cells in a suspension. About 25% of the embryos developed to morula or blastocysts. Upon transfer to recipient cows by non-surgical methods, about 30% of these embryos resulted in pregnancies.

WHAT IS CLAIMED IS:

1. A method to culture bovine embryos in vitro comprising steps of

a) collecting early stage embryos;

b) placing the early stage embryos in a suspension culture system of buffered balanced salt solution with a carbohydrate energy source medium and bovine oviductal epithelial cells;

c) holding the suspension system at about 38°C and about 5% CO_2 and 95% air humidified atmosphere; and

d) culturing the embryos until morula or blastocyst stage.

2. A method to culture bovine embryos in vitro of claim 1 wherein in step (a) the examining of the early stage embryos and selecting 1- to 2-cell embryos for culturing.

3. A method to culture bovine embryos of claim 1 including the step of transferring the cultured embryo to a recipient cow.

4. A method to culture bovine embryos of claim 1 including the step of covering the suspension system under a film after step (b).

5. A method to culture bovine embryos in vitro comprising the steps of

a) collecting early stage embryos;

b) placing the embryos in a mixture of bovine or ovine oviductal fluid, buffered balanced salt solution with a carbohydrate energy source medium and bovine oviductal epithelial cells in a suspension system;

c) holding the suspension system at about 38°C and about 5% CO_2 and 95% air humidified atmosphere; and

d) culturing the embryos until morula or blastocyst stage.

6. A method to culture bovine embryos in vitro of Claim 5 including the step of transferring the cultured embryo to a recipient cow.

7. A method to culture bovine embryos of Claim 5 including the step of covering the suspension system under a film after step (b).

8. A method to culture bovine embryos in vitro comprising the steps of

a) collecting early stage embryos;

b) placing the embryos in a mixture of bovine or ovine oviductal fluid, buffered balanced salt solution with a carbohydrate energy source medium supplemented with amino acids and bovine oviductal epithelial cells in a suspension system;

c) holding the suspension system at about 38°C and about 5% CO_2 and 95% air humidified atmosphere; and

d) culturing the embryos until morula or blastocyst stage.

9. An in vitro culture system for bovine embryos comprising a mixture of a buffered balanced salt solution with a carbohydrate energy source and bovine oviductal epithelial cells in a suspension system.

10. An in vitro culture system of bovine embryos of claim 9 wherein said buffered balanced salt solution with carbohydrate energy source is supplemented with amino acids.

11. An in vitro culture system for bovine embryos comprising a mixture of a buffered balanced salt solution with a carbohydrate energy source with oviductal fluid selected from the group of bovine or ovine and oviductal epithelial cells in a suspension system.

12. An in vitro culture system for bovine embryos of claim 11 wherein the buffered balanced salt solution with a carbohydrate energy source medium and oviductal fluid is in a 1:1 mixture.

13. An in vitro culture system for bovine embryos of claim 11 wherein the oviductal epithelial cells are about 5% to 15% volume of the fluid media.

14. An in vitro culture system for bovine embryos of claim 11 wherein the buffered balanced salt solution with a carbohydrate energy source is supplemented with amino acids.

15. An in vitro culture system for bovine embryos of claim 11 wherein said buffered balanced salt solution with carbohydrate energy source is supplemented with amino acids.

INTERNATIONAL SEARCH REPORT

International Application No PCT/US 88/04700

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [6]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC[4]: C 12 N 5/00

II. FIELDS SEARCHED

Minimum Documentation Searched [7]

Classification System	Classification Symbols
IPC[4]	C 12 N; A 01 N; A 61 K

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT [9]

Category [10]	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No. [13]
X	Theriogenology, vol. 27, no. 1, 1987, W.H. Eyestone et al.: "Co-culture of early bovine embryos with oviductal epithelium", page 228 see the whole article cited in the application	1,3,4,9,10
Y		5-8,11-15
	--	
X	J. Reproduction and Fertility, vol. 81, no. 1, 1987, F. Gandolfi et al.: "Stimulation of early embryonic development in the sheep by co-culture with oviduct epithelial cells", pages 23-28 see the whole article	1-4,9,10
Y		5-8,11-15
	--	
Y	J. Reproduction and Fertility, vol. 30, 1972, H.R. Tervit et al.: "Successful culture in vitro of sheep and cattle ova", pages 493-497 ./.	5-8,11-15

* Special categories of cited documents: [10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
25th May 1989	10 JUIL. 1989
International Searching Authority	**Signature of Authorized Officer**
EUROPEAN PATENT OFFICE	P.C.G. VAN DER PUTTEN

International Application No. PCT/US 88/04700

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
	see page 493, lines 29-34	
A	--	1-4,9,10
A	J. Reproduction and Fertility, vol. 72, no. 2, 1984, Journals of Reproduction and Fertility Ltd, S. Camous et al.: "Cleavage beyond the block stage and survival after transfer of early bovine embryos cultured with trophoblastic vesicles", pages 479-485 see the whole article cited in the application	1-15